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REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 CFR 1.322 AND UNDER 37 CFR 1.323
Docket No. CGS-101T
Patent No. 6,794,140

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Andrew Simon Goldsborough
Issued	:	September 21, 2004
Patent No.	:	6,794,140
For	:	Isolation of Nucleic Acid

Mail Stop Certificate of Corrections Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION
UNDER 37 CFR 1.322 (OFFICE MISTAKE)
UNDER 37 CFR 1.323 (APPLICANT'S MISTAKE)

Sir:

A Certificate of Correction (in duplicate) for the above-identified patent has been prepared and is attached hereto.

In the left-hand column below is the column and line number where errors occurred in the patent. In the right-hand column is the page and line number in the application where the correct information appears.

Patent Reads:	**Application Should Read:**
Column 2, Line 59: "et al. (1995)"	Page 5, Line 6: --et al., (1995)--
Patent Reads:	**Application Reads:**
Column 3, Line 32: "(1077)"	Page 6, Line 17: --(1977)--

Patent Reads:	**Application Should Read:**
Column 3, Line 50: "conditions it will be should be possible"	Page 7, Lines 3-4: --conditions it will be/should be possible--
Column 3, Line 61: "etc"	Page 7, Line 17: --etc.--
Patent Reads:	**Application Reads:**
Column 4, Line 2: "RNAsuch"	Page 7, Line 27: --RNA such--
Patent Reads:	**Application Should Read:**
Column 4, Line 32: "pyrrolidonone"	Page 8, Line 27: --pyrrolidone--
Column 4, Line 33: "Acids. Res. 25:3925) Capillary	Page 8, Line 29: --Acids Res. 25:3925). Capillary--
Column 8, Line 9: "C_1-C_{36} C_1-C_{36} aminoalkanoyl"	Page 16, Line 33: --C_1-C_{36} aminoalkanoyl--
Patent Reads:	**Application Reads:**
Column 9, Line 52: "heads"	Page 20, Line 18: --beads--
Column 9, Line 56: "head"	Page 20, Line 22: --bead--
Column 9, Line 59: "of the"	Page 20, Line 25: --to the--
Column 10, Line 20: "reacts the"	Page 21, Line 24: --reacts with the--
Column 10, Line 24: "reaction"	Page 21, Line 27: --reactive--
Column 10, Line 27: "efficiently"	Page 21, Line 32: --effectively--

Column 10, Line 65: "reaction"	Page 23, Line 6: --reactant--
Column 11, Line 23: "glass glide"	Page 23, Line 35: --glass slide--
Column 11, Line 61: "PCPB"	Page 25, Line 9: --BCPB--
Column 12, Line 12: "solid"	Page 25, Line 29: --solvent--
Column 12, Line 13: "catalyst"	Page 25, Line 31: --catalysed--
Column 12, Lines 62-63: "RNA temperature"	Page 27, Line 18: --RNA template--
Column 13, Line 26: "heads"	Page 28, Line 16: --beads--
Patent Reads:	**Application Should Read:**
Column 14, Line 4: "30 000 – 100 000"	Page 30, Line 1: --30,000 – 100,000--
Patent Reads:	**Application Reads:**
Column 14, Lines 56-57: "125 M NaCl"	Page 31, Line 32: --125 mM NaCl--
Column 14, Line 60: "PCPB"	Page 32, Line 3: --BCPB--
Column 14, Line 66: "reactively"	Page 32, Line 10: --reactivity--
Column 15, Line 11: "PCPB"	Page 32, Line 24: --BCPB--

Patent Reads:	**Application Should Read:**
Column 15, Line 20: "agel"	Page 33, Line 1: --a gel--
Patent Reads:	**Application Reads:**
Column 15, Line 49: "RNA form"	Page 33, Line 34: --RNA from--
Column 15, Line 55: "($ClOC(CH_2)_2COCl$)"	Page 34, Line 6: --($ClOC(CH_2)_4COCl$)--
Column 15, Line 56: "used is"	Page 34, Line 7: --used to--
Patent Reads:	**Application Should Read:**
Column 17, Line 27: "1999 s"	Page 37, Line 22: --1999 as--
Patent Reads:	**Application Reads:**
Column 17, Line 66: "or other"	Page 38, Line 31: --or others--
Column 19, Line 47: "betyl-,"	Page 42, Line 21: --octyl-,--
Patent Reads:	**Application Should Read:**
Column 19, Lines 63-64: "beads in of 1.7M"	Page 43, Line 4: --beads in 1.7M--
Patent Reads:	**Application Reads:**
Column 20, Line 12: "biding"	Page 43, Line 20: --binding--
Column 20, Line 16: "octyl or"	Page 43, Line 25: --octyl to--

Column 21, Line 9: "N-methylsatoic"	Page 45, Lines 18-19: --N-methylisatoic--
Column 21, Line 25: "Proemga"	Page 46, Line 1: --Promega--
Column 21, Line 29: "SDS three times"	Page 46, Line 5: --SDS and three times--
Column 21, Line 56: "BDPB"	Page 46, Line 34: --BCPB--
Column 26, Lines 11-12: "(pH 8.4 to 24° C.)"	Page 54, Line 30: --(pH 8.4 at 24° C.)--
Column 26, Line 19: "6.0 mM"	Page 55, Line 6: --60 mM--
Column 26, Line 30: "perchlorate to SDS"	Page 55, Line 17: --perchlorate or SDS--.

A true and correct copy of pages 6, 7, 20, 21, 23, 25, 27, 28, 31-34, 38, 42, 43, 45, 46, 54, and 55 of the specification as filed, which support Applicant's assertion of errors on the part of the Patent Office, accompany this Certificate of Correction.

The Commissioner is authorized to charge the fee of $100.00 for the amendment to Deposit Account No. 19-0065. The Commissioner is also authorized to charge any additional fees as required under 37 CFR 1.20(a) to Deposit Account No. 19-0065. Two copies of this letter are enclosed for Deposit Account authorization.

Approval of the Certificate of Correction is respectfully requested.

Respectfully submitted,

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FCE/sl

Attachments: Certificate of Correction; copies of pages 6, 7, 20, 21, 23, 25, 27, 28, 31-34, 38, 42, 43, 45, 46, 54, and 55 of the specification

degradation occurs during the analytical process. However, RNA (tRNA) up to 142 nucleotides (Nordhoff et al., (1993) Nucleic Acids Res. 21:3347; Gruic-Sovulj et al., (1997) Nucleic Acids Res. 25:1859; Tolson and Nicholson (1998) Nucleic Acids Res. 26:446) and double stranded DNA up to 500 base-pairs (Bai et al. (1995) Rapid Comm. Mass Spectrom. 9:1172; Taranenko et al., (1998) Nucleic Acids Res. 26:2488; Ausdall and Marshall (1998) Anal. Biochem. 256:220) have been measured using MALDI mass spectrometry (for reviews see; Smith (1996) Nat. Biotech. 14:1084; Murray (1996) J. of Mass Spectrom. 31:1203. Phosphate (Schuette et al., (1995) J. Pharm. Biomed. Anal. 13:1195; Sinha et al., (1994) Nucleic Acids Res. 22:3119) and chemically modified oligonucleotides (Potier et al., (1994) Nucleic Acids Res. 22:3895) have also been measured using mass spectrometry.

Although there is a molecular weight limitation to a few hundreds of nucleotides when using mass spectrometry, it provides a simple, automated means to accurately determine the exact molecular weight and therefore the percentage modification of a polynucleotide. Optimisation relies on a number of factors such as the type of mass spectrometry being carried out (electro-spray, MALDI-TOF etc), the method used to purify the modified RNA from the modification reaction, the size of the polynucleotide, the ionisation matrix used, the method used to remove cations from the RNA, positive or negative ion mode and the voltage strength used (Fenn et al., 1989) Science 246:64). Capillary high performance liquid chromatography can be used prior to mass spectrometry of RNA because desalting and other purification steps are not required prior to ionisation (Taniguchi and Hayashi (1998) Nucleic Acids Res. 26:1481).

To measure the molecular weight and hence the percentage modification of polynucleotides consisting of thousands of nucleotides requires a different approach. In certain situations where it is preferable to measure the percentage modification of the polynucleotide using more precise means a degradative step may be employed followed by an analytical process. It is expected that degradation of the modified polynucleotide using chemical or enzymatic means will, depending on the method used leave the 2'-OH modification attached to the ribose sugar allowing the amount of modification to be established by mass spectrometry or high performance liquid chromatography (HPLC). HPLC and gas chromatography analysis of nucleotides has been described (Gehrke and Patel (1977) J. Chromat. 130:103; Iwase et al., (1975) J. Chromat. 106:213; Kemp et al., (1982) J. Chromat. 241:325).

In order to establish the percentage of nucleotides that are modified, degradation of the polynucleotide should follow the modification reaction. Methods have been described for enzymatic cleavage methods employing ribonucleases RNase T1, RNase A, RNase U2, RNase PhyM, RNase CL3, nuclease S7 and cusativin, chemical cleavage methods using sulfuric acid (Jones et al., (1994) RNA Isolation and Analysis, chapter 3, Bios Scientific Publishers, Oxford) and physical methods using post source decay (Hahner et al., (1997) Nucleic Acids Res. 25:1957; Taniguchi and Hayashi (1998) Nucleic Acids Res. 26:1481; Kirpekar et al., (2000) RNA 6:296).

It will be understood that the 2'-OH modification

may inhibit degradation of the polynucleotide. However, by empirically determining the sensitivity of the modified RNA to a range of conditions it will be should be possible in most cases to select conditions that are suitable for chain cleavage. For example, it has been found that acetylated RNA is readily cleaved by nuclease Bal 31. Whilst alkali cleaves acetylated RNA it also results in acetyl cleavage so unless the amount of cleaved acetyl groups is measured by mass spectrometry, acetylated nucleotides will not be detected. For example, acid cleavage of the modified polynucleotide can be used for base sensitive modifications, whilst base cleavage can be used for acid sensitive modifications. It will also be understood that other degradation products such as dinucleotides, trinucleotides etc will also be suitable for measuring the percentage modification of the polynucleotide. Whether it is the nucleotide, dinucleotide or larger fragments that are being measured, in each case it is the ratio of the number of fragments bearing a modification compared with the number of fragments not bearing a modification that provides the percentage modification.

Other methods that are capable of measuring high molecular weight RNA such as analytical ultracentrifugation to find the sedimentation coefficients (Svedberg units) are imprecise, require large amounts of starting material and are dependent on the conformation of the RNA (for review, see Jones et al., (1994) RNA Isolation and Analysis, chapter 3, Bios Scientific Publishers, Oxford). Despite these drawbacks, analytical ultracentrifugation using denaturing sucrose or

isokinetic gradients may be useful to measure very large molecular weight changes in abundant RNA samples.

It is now much more common to measure the molecular weight of polynucleotides using electrophoretic separation in polyacrylamide or agarose gels. Detailed descriptions of the preparation, use and handling of electrophoresis gels is described in several publications (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH; Jones (1995) Gel Electrophoresis: Nucleic Acids Essential Techniques, Wiley). Denaturing gels are preferred to non-denaturing gels because they reduce conformational effects providing a means to measure the true molecular weight of the linear polynucleotide (Jones (1995) Gel Electrophoresis: Nucleic Acids Essential Techniques, page 47, Wiley). There are a variety of denaturants that can be used such as DMSO (50-90%), glyoxal (10-30%), formaldehyde (3% w/v), formamide (50-98%), heat (60-80°C), methyl mercuric hydroxide (3-5mM), sodium iodoacetate (10mM), 2-pyrrolidone (5%) and urea (6-8mM). It is known that incomplete denaturation of the polynucleotide leads to anomalous migration so that more than one denaturing condition may be required such as 8M urea plus 5% pyrrolidonone or 8M urea run at 60°C (Rosenblum et al., (1997) Nucleic Acids. Res. 25:3925) Capillary electrophoresis provides an excellent means to carry out such molecular weight determinations and suitable methods have been described for RNA (Engel and Dieguez-Lucena (1993) Nucleic Acids Res. 21:759).

Comparative measurements of polynucleotide migration

protein such as streptavidin. Another useful affinant comprises a primary amino group capable of chemically reacting with an immobilised partner such as succinimide.

In one arrangement, the substituent comprises a hydrophobic substituent so that the RNA may be modified to render it more hydrophobic for the purpose of isolation. Both DNA and RNA are relatively hydrophilic molecules. It is quite difficult to separate RNA from DNA because their physical characteristics are similar. By increasing the hydrophobicity of RNA relative to DNA it is possible to improve the separation of the two types of nucleic acid. This is useful for either removing contaminating RNA from a DNA sample or removing contaminating DNA from RNA. For example it is important to remove bacterial RNA from a plasmid preparation prior to restriction enzyme analysis so that small DNA fragments are not obscured by the co-migrating RNA during agarose gel electrophoresis. Alternatively it is important to remove traces of DNA such as genomic DNA or viral DNA from cellular or viral RNA. Such DNA contamination often leads to false positives following RT-PCR amplification.

The hydrophobic substituent typically comprises a substituent, OR, wherein R comprises C_1-C_{36} alkyl; C_1-C_{36} alkenyl; C_1-C_{36} alkynyl; C_1-C_{36} haloalkyl; C_1-C_{36} aminoalkyl; C_1-C_{36} alkoxyalkyl; C_1-C_{36} alkylthioalkyl; C_1-C_{36} alkoxyalkoxyalkyl; C_1-C_{36} haloalkoxyalkyl; C_1-C_{36} aminoalkoxyalkyl; C_6-C_{36} aryl; C_6-C_{36} alkylaryl; C_6-C_{36} arylalkyl; C_6-C_{36} arylalkenyl; C_1-C_{36} alkanoyl; C_1-C_{36} alkenoyl; C_1-C_{36} haloalkenoyl; C_1-C_{36} haloalkanoyl; C_2-C_{36} haloformylalkanoyl; C_1-C_{36} C_1-C_{36} aminoalkanoyl; C_1-C_{36} azidoalkanoyl; C_1-C_{36} carboxyalkanoyl; C_1-C_{36} carboxyalkenoyl; C_1-C_{36} carboxyalkynoyl; C_1-C_{36}

Advantages of using RNA covalently bound to a solid phase include ease of manipulation, purification, automation and compatibility with many important life science applications such as diagnostics and detecting gene expression. It is also a convenient means to limit the amount of modification occurring on the RNA polynucleotide because the reactive groups are spatially restricted. RNA modified at only a few 2'-OH positions may retain important biological functions such as serving as a template for protein translation, hybridisation and serving as a template for a polymerase.

There are many potentially useful solid phase reactants (see review by Akelah and Sherrington (1981) Chem. Rev. 81:557) such as other carboxylic acid derivatives including acid halides and acid anhydrides. Benzoyl chloride polymer bound (BCPB) beads are commercially available (Fluka, USA) with high densities of benzoyl chloride groups (approximately 2.1mmol/g of resin). RNA can be covalently attached to the benzoyl chloride solid support via an ester linkage between the BCPB bead and the 2'-OH group. Under appropriate reaction conditions, the 2'-OH groups of the RNA will react with the benzoyl chloride and become covalently coupled to the solid phase. Due to the physical separation of the benzoyl chloride groups, it is highly unlikely that all (100%) of the 2'-OH groups of a polynucleotide will be modified, rather only one or a few will be modified per polynucleotide. In order to immobilise RNA, it is sufficient that only one 2'-OH group reacts with the solid phase reagent.

Other solid phase reactive groups that may be suitable for immobilising RNA could include acid anhydrides. In the case of acid anhydrides it is important that the half of the acid anhydride attached to the solid phase is the part that reacts with the 2'-OH group thereby immobilising it. Otherwise, the result will be modified RNA in solution. In order to overcome this potential problem, an asymmetric acid anhydride may be attached to the solid phase so that the half of the acid anhydride attached to the solid phase reacts preferentially with the 2'-OH group resulting in immobilised RNA. Other examples include the use of cyclic anhydrides such as isatoic anhydride polymer bound (Cat 17344, Fluka, USA). However, it is simpler to employ carboxylic acid derivatives such as the acid halides (e.g. benzoyl chloride polymer bound), acid cyanides (e.g. benzoyl cyanide polymer bound) or acid imidazoles (e.g. N-benzoyl imidazole polymer bound) because the outcome of the reaction will be an ester linkage between the solid phase and the polynucleotide.

It will be understood that the solid phase reactant need not be limited to carboxylic acid derivatives, rather any reactant that reacts with the 2'-OH group in a regiospecific manner is suitable. The amount of particles required to immobilise a given amount of RNA will depend on several factors such as the density of the reactive groups and the proportion of the reactive groups that will react with the 2'-OH. Reactive groups that are buried, for example within the resin will be unable to react with a large molecule like RNA and they will therefore be effectively unreactive. The precise RNA binding capacity for a particular solid phase reactant will require empirical tests. If steric hindrance occurs between the polynucleotide and the reactive group due to

The 'particle' or macromolecular structure may be incorporated into many forms such as a strip, a fibre, a matrix, a membrane, a filter, a column, a bead, a resin, a vessel wall, a pipette tip, a gel or a plate or an etched silicon device.

The reactant may be covalently attached to the solid phase using for example divinyl benzene (DVB) or associated by another means such as ionic interaction or hydrogen bonding so that in any case the RNA is firmly held to the solid phase during washing and analysis. However covalent attachment is preferred because there will be less tendency for the reactant to dissociate from the solid phase. The solid phase reactant may be incorporated into a microdevice or vessel such as those made from etched silicon and the solution containing the RNA passed over or through the region containing the reactant so that RNA immobilisation occurs in a specific region of the solid phase. This process may be automated in order to detect for example RNA viruses such as HCV and HIV in blood or body fluids. The immobilised sample might then be used for any number of down stream applications such as hybridisation, RT-PCR, TMA or NASBA.

Alternatively, the solid phase reactant may be incorporated into a blood collection device such that the RNA components of the blood are immobilised during the blood collection process. The solid phase reactant may also be coated or attached to a glass slide in many small (preferably less than $1mm^2$) discrete regions. A single RNA sample from, for example a tissue source may be added (in a suitable solvent and catalyst) to one of the discrete regions thereby localising the RNA to a discrete position of the glass slide. The process may

variety of quantitative assays such as hybridisation with labelled probes or serve as a template for RT-PCR. In the latter case, it has been found that BCPB beads have the advantage of being compatible with both the components of the reverse transcription and PCR reactions. Other purification materials such as silica beads inhibit these reactions.

A marked advantage of using BCPB beads compared with silica beads to purify RNA is that there is no elution step required to separate the RNA from the solid phase. If silica beads are added to the reverse transcription reaction they would bind both the template RNA, primers and a proportion of the enzyme leading to reduced or total inhibition of cDNA synthesis. It has been found that following RNA reaction, BCPB beads can be added directly into both the reverse transcription and PCR reactions with no such inhibition. BCPB beads may also find utility for other types of reactions such as LCR and NASBA. Adding the beads to the reaction simplifies manipulation and ensures that all the captured RNA is transferred into the reaction. The bead format could prove useful for automated systems in the diagnostic field. Thus BCPB beads are especially preferred in the present invention.

Solvents

When RNA is modified using acetic anhydride in a basic solvent such as triethylamine with DMAP, the modification of the 2'-OH groups proceeds so quickly that base catalysed cleavage of the RNA is insignificant. However, when the reactant is attached to a solid phase such as the BCPB, it is unlikely that all the 2'-OH groups can be modified due to the physical immobilisation of the reactant. Therefore, even after extensive incubation

washing with aqueous solutions. Preferred bead sizes are those that provide a maximum surface area for reaction to occur with the RNA. Buried reactive groups will not be accessible to the RNA, therefore beads and particles with a small diameter (large surface area to volume) are preferred. However, in order to collect the beads from the liquid phase it is necessary that they are not so small that they cannot be easily pelleted by centrifugation, collected by filtration or selected by other means. Commercially available BCPB beads with a mesh size of 100-300 are readily pelleted using a centrifugal force of 1500g for 5sec.

Length of RNA suitable for RT-PCR

Due to the physical separation of the reactive groups on the solid phase, a single RNA template may be linked to the solid phase via only one or a few 2'-sites. If the RNA template is relatively long and the sequence to be amplified relatively short, there will only be a small number of RNA templates that are attached within the sequence to be amplified. These templates will probably not be copied by the enzyme because the2'-modification may block the passage of the enzyme. The HCV RNA genome is approximately 10,000 nucleotides long, if the PCR primers span a region of 100 bp, only 1% of the polynucleotides will not amplify due to the2'-modification. However, if the RNA template is shorter, for example 1000 nucleotides and the PCR primers span 1000 bp, then none of the RNA will serve as a template for RT-PCR. It is therefore preferable to PCR amplify only short regions of the RNA. Furthermore, the reverse transcriptase primer should be as close to the 3' PCR primer as possible, preferably being of identical sequence. However, for RT-PCR analysis of mRNA where the cDNA synthesis has to be primed using an oligo (dT)

primer, it is preferable to use PCR primers that amplify sequences close to the 3′ end of the mRNA. Alternatively the RNA may be released from the solid phase using chemical or enzymatic deprotection, for example by alkali or KCN cleavage of the ester linkage before RT-PCR.

DNA Binding

Under specific conditions, some DNA will bind non-specifically to the beads probably through hydrophobic interactions with the BCPB bead. Such interactions can be reduced by using DMF, pyridine, ethyldiisopropylamine (EDPA), triethylamine or DMSO as the reaction solvent instead of THF. Non-specific DNA binding can be removed by one or more washes in 10% SDS. However, DNA may react with BCPB beads covalently via either the3′ or 5′-OH groups. Less than 3% of a labelled DNA sample became covalently attached when DMSO was used as a solvent for the BCPB beads compared with 14% with THF as a solvent.

Protein binding and diagnostic systems

In a similar manner to DNA, some protein will bind to BCPB. It was found that adding detergents to a THF solvent containing ^{35}S-labelled cellular protein enhanced the amount of protein binding to the beads. This may be caused by the detergent unfolding the protein and therefore exposing more hydrophobic residues to the BCPB. The greatest enhancement was seen with a final concentration of 0.025% TWEEN/NP-40 detergents, THF and protein were mixed and incubated 3 min at 22°C with 3mg of BCPB. Protein binding could be reduced by 50% by pre-reacting the protein with acetyl chloride. Protein binding to BCPB was not reduced if the reactivity of the

cut-off of 30 000 -100 000 daltons such as CENTRICON - 100, CENTRIPLUS-100 (Amicon, US). Filters with pore sizes above 0.01µm such as ISOPORE 0.05µm polycarbonate membranes (Millipore, US) should generally capture most virus particles from blood serum whilst allowing most proteins to pass through. Such collected virus particles could then be added to the reactant-solid phase such as BCPB beads.

The volume of a body fluid such as serum may be reduced prior to addition of the reactant-solid phase using a CENTRIFREE filtration device. The retained sample containing protein and viral RNA could then be added to a solvent containing the reactant-solid phase such as BCPB beads.

Blood contains approximately 70mg/ml of protein and a large component of this is albumin and immunoglobulins. Means to reduce serum proteins include the addition of immobilised *S. aureus* protein A or thiophilic resin (Sigma, US) which bind immunoglobulins, a major component of blood protein. Protein A linked to a solid phase such as a bead or thiophilic resin would provide a facile means to reduce blood protein concentration.

Enhancing the Reaction

The RNA binding reaction may be enhanced by reducing the volume of the reaction to a minimum required to keep the RNA soluble and in contact with the beads. Approximately 10 µl of solvent is required to immerse 1-3 mg of BCPB. It was found that adding plasmid DNA to a

final concentration of 25ng per microlitre enhanced RNA binding, possibly by increasing the apparent concentration of the RNA. Other volume excluding compounds such as polyethylene glycol may also enhance RNA binding.

Addition of detergents and chaotropes

In order to increase specific RNA binding whilst reducing protein binding, detergents were added to a reaction containing BCPB beads in different solvents. It was found that the addition of a final concentration of 1% SDS in DMSO or THF increased the amount of RNA bound to BCPB by 33% and 42% respectively whilst 1% SDS in DMF or toluene markedly reduced RNA binding. It was also found that except for toluene, a final concentration of 1% SDS increased the amount of ^{35}S labelled cellular protein bound to BCPB in DMF, DMSO and THF by 6.8, 2.3 and 1.85 times respectively that of a parallel reaction containing no added SDS. EDPA, pyridine or water led to low levels of RNA binding.

It was found that addition of 150mM sodium perchlorate (final concentration) to 40µl DMSO containing 3mg BCPB beads, 10µg BSA and 20ng of radiolabelled RNA increased the amount of RNA bound by 2.3 fold. Even lower sodium perchlorate concentrations of 1.5mM and 15mM (final concentration) increased RNA binding by 1.11 and 1.33 fold respectively. This effect was not caused by the high salt concentration because a parallel reaction containing 125mM NaCl instead of sodium perchlorate bound 30% less RNA.

The addition of sodium perchlorate also increases the amount of protein bound to the beads. 40µl of DMSO containing 3mg BCPB beads was mixed with 1µl of ^{35}S labelled cellular protein with either 1µl of water or with 1.5mM, 15mM or 150mM sodium perchlorate (final concentration), protein binding increased by 0, 1.38 and 1.5 times respectively with sodium perchlorate.

However, 600mM urea or 400mM guanidine thiocyanate in THF reduced BCPB reactivity by 39% and 73% respectively. The addition of chaotropes and/or detergents is important because if RNA is to be isolated intact from cells, blood or other biological material, it is necessary to inhibit ribonuclease activity. One way to do this is to use a slight excess of chaotrope and/or detergent and then add this to the solid phase reactant in a suitable solvent system thereby immobilising the RNA. The solid phase may then be washed to remove contaminating proteins and DNA. Suitable chaotropes may include guanidinium chloride, guanidinium thiocyanate, sodium iodide, sodium perchlorate and sodium trichloroacetate.

An aliquot of BCPB stored at 22°C was opened over the course of 3 weeks and exposed to the air approximately 30 times. No reduction in reactivity was noted. BCPB is thus sufficiently stable for the present applications. Water in excess of 7.5% vol/vol in THF reduces the reactivity of BCPB. The preferred water content is 3µl or less in 40µl of THF with 1mg of BCPB.

To reiterate, in addition to the preferred BCPB beads, the solid phase could include a particle, a bead, a

membrane, agel, a slide or cover slip, an etched silicon surface, a fibre, a filter, a capillary, a tube, a vessel or a multi-vessel plate such as a 96 well plate. It will be apparent that the use of a solid phase improves handling throughput and accuracy when the RNA immobilization and analysis is automated. In this context, paramagnetic particles are favoured for their handling properties. Other preferred solid phases are those which comprise OH groups, e.g. compounds of silicon and oxygen, such as silica particles or a glass. Nucleic acid purification using silica beads is well known and widely practiced both in diagnostic and life science research. Methods of purifying RNA have been described in US patent 5,234,809. Briefly the method involves binding the nucleic acid sample to silica beads in a chaotropic agent such as urea and then washing the beads in a high salt wash before it is eluted into water. The modified RNA used in the present invention has been tested for its ability to bind and be released from silica beads using a silica bead purification kit (Qiagen, Germany) and found to be particularly suited to this type of separation method (see Examples).

As mentioned above, according to US Patent 5,234,809, the purification of nucleic acids from biological samples involves mixing silica particles with a chaotropic salt containing the sample. Under these conditions the nucleic acid binds reversibly to the silica. During attempts to find new methods to separate modified RNA from the reaction components, it has been discovered that modified RNA will readily bind to silica beads, e.g. in the presence of 95% organic solvent. This unexpected result suggests that silica particles are suitable to bind modified RNA from a variety of organic solvents.

Agents may be employed to aid in binding the RNA to the solid phase. Bifunctional acid halides, e.g. acid chlorides, are commercially available that have effectively two reactive groups per molecule. Reagents such as sebacoyl chloride ($ClOC(CH_2)_8COCl$), adipoyl chloride ($ClOC(CH_2)_4COCl$) and glutaryl chloride ($ClOC(CH_2)_3COCl$) can be used to immobilise RNA by covalent modification of the 2'-OH group with consequent attachment to a solid phase. The bifunctional acid chloride can either be reacted with the RNA and then with a solid phase, or preferably with a solid phase and then the RNA sample. In both cases, the solid phase is conveniently BCPB beads, or one which has hydroxyl groups such as glass and other silicon dioxide compounds. It is known that boiling concentrated HCl exposes hydroxyl groups on the surface of glass. Such prepared glass can then be reacted with an excess of the bifunctional acid chloride, the unreacted acid chloride is removed by washing in solvent such as THF and then the RNA sample added and allowed to react with the immobilised acid chloride, thereby becoming immobilised via the acid chloride to the glass solid phase. Bifunctional acid chlorides are preferred that have an extended hydrocarbon spacer between each end of the molecule such as with sebacoyl chloride (Cat. No. 84848 Fluka, USA) because these are likely to reduce steric hindrance between the RNA and the solid phase and therefore improve the reaction.

Modification of RNA whilst bound to silica beads

Nucleic acids become bound to the surface of silica beads, therefore tests were carried out to find out whether RNA can be modified whilst bound to the silica beads. Although it may be expected that some of the 2'-OH groups are protected from modification due to

tip such as those commonly used to measure 1µl, 10µl, 200µl or 1 ml volumes. In either case, capture, washing and elution of the modified RNA is improved because the time required to separate the modified RNA from the contaminants is reduced.

Multiple RNA samples could be purified in parallel by the use of devices bearing multiple capture surfaces. An example would be a 96-well plate whereby each well is hydrophobic and suitable for capture, washing and elution of one modified RNA sample. A further example would be a vessel or chamber suitable for the modification reaction and in addition the capture, washing and elution of the RNA sample. In this way, both the modification and purification is carried out in the same vessel or chamber improving sample throughput and productivity. The modification, capture, washing and elution of the RNA sample could be automated and involve robots.

Methods of treatment of the RNA are described in further detail in UK patent applications, nos. 9910154.5 entitled POLYNUCLEOTIDES published 30 June 1999 s GB9910154A, 9910157.8 entitled POLYNUCLEOTIDES published 30 June 1999 as GB9910157A and 9910156.0 also entitled POLYNUCLEOTIDES published 30 June 1999 as GB9910156A. Each of these copending patent applications was filed 30 April 1999 in the name of the same applicant. For example, Example 6 of each of these copending patent applications sets out one preferred method of modifying RNA involving a DMAP-catalysed acetylation reaction. The methodology in that Example may be modified using instead of acetic anhydride, anhydrides of longer chain length including butyric or pentanoic anhydrides as set out in Example 54 of each of the copending applications. In addition,

Example 1 of each of these copending patent applications sets out a method of modifying a total cellular RNA population and selection of the mRNA fraction.

It will also be apparent to those skilled in the art that a crude cellular or tissue lysate consisting of RNA, DNA, protein and lipids etc. may serve as the sample for the modification reaction. In this case, the reactant may modify not only the 2'-OH group of the RNA chain but also the hydroxyl bearing side chains of the amino acids tyrosine, serine or threonine of proteins. It will be apparent that such a reaction is beneficial because it will lead to the deactivation of cellular nucleases and therefore allow the selection of RNA in a more intact form. Furthermore, cells could be disrupted in the presence of the reactant so that nucleases are immediately inactivated on release from the cell and the RNA is immediately modified and therefore protected from any remaining nucleases. In this latter case, increased concentrations of the reactant in the reaction may be required in order to inactivate the nucleases fully as well as to modify fully the 2'-OH groups of the RNA sample.

The treated DNA and RNA sample in a high salt buffer (e.g. 1-5 M ammonium sulphate, preferably 10 mM phosphate, pH 7.0, 1.5 M ammonium sulphate) is passed over a hydrophobic column as is commonly used for reverse phased liquid chromatography. For example reversed phase packings based on silica may have bonded hydrocarbon chains of C4, C8 or C18 or others may be based on polystyrene (e.g. POROS® and OLIGO R3, PerSeptive Biosytems, USA) may be used under appropriate conditions. Further alternatives could

and other cellular components by mixing and agitating by use of a vortex etc. the mixture with appropriate solvents such as (in order of decreasing polar property) pentane, toluene, chloroform, THF, DMSO or methanol. Under ideal conditions it would be expected that all the modified RNA would partition into the hydrophobic phase whilst non-RNA contaminants would remain in the aqueous phase. Simple separation of the hydrophobic phase by pipetting followed by ethanol precipitation or evaporation of the solvent would provide a highly purified source of modified RNA.

Example 3

Differential Interaction with Immobilised hydrocarbon chains

Direct interaction between RNA molecules bearing hydrophobic groups and hydrophobic groups attached to a solid support would provide an efficient means to separate RNA from contaminants. Hydrophobic solid supports include ethyl-, propyl-, butyl-, pentyl-, hexyl-, octyl-, decyl and dodecyl-agarose affinity chromatography media (Catalogue ref. AAF-8, Sigma-Aldrich Chemicals). Interaction between the modified RNA and the media should be stronger with longer chain lengths attached to either the RNA or immobilised support. By careful choice of the binding solution it is possible to selectively bind RNA to the beads whilst the contaminants such as DNA are retained in the binding solution. The polarity of the solvent used for binding and washing, the type and concentration of detergent, temperature of interaction and carbon chain length used will all influence the effectiveness of the purification.

60µl of ethyl-agarose or dodecyl-agarose beads (Sigma, USA) were washed twice in 200µl of 1.7M ammonium sulphate and collected by centrifugation at 3000g for 5 seconds between washes. 40µl (20%) of the beads in of 1.7M ammonium sulphate were added to 300ng of isatoic anhydride labelled RNA. It was found that the fluorescent isatoic anhydride modification could be used to monitor binding to the hydrophobic beads under ultra-violet light. On addition to the beads, fluorescent RNA moved from the solvent to the beads within a few seconds demonstrating interaction between the modified RNA and the hydrophobic surface. It was also found that the interaction between the modified RNA and dodecyl-agarose was very strong: 1% TWEEN/1% TRITON X-100, 6M urea,100% ethanol or loading the sample in a well of an agarose electrophoresis gel and subjecting the bead-RNA complex to 100V for 15min. failed to displace the labelled RNA from the dodecyl-agarose bead whilst 50mM sodium phosphate buffer removed most of the modified RNA from ethyl-agarose beads. An intermediate binding affinity between ethyl and dodecyl was found in the ease of releasing modified RNA from propyl, pentyl and octyl-agarose. Overall, the strength of the hydrophobic interaction is proportional to carbon chain length increasing from ethyl, propyl, pentyl, octyl to dodecyl-agarose.

This demonstrates that as expected, the strength of the hydrophobic interaction between modified RNA and the hydrophobic bead is dependent on the chain length and therefore hydrophobicity of the bead. The ease with which modified RNA may be removed from the hydrophobic surface is therefore a function of the hydrophobicity of both the modification at the 2′ position of the RNA and the nature

modification reaction

2mg of BCPB beads were added to 40µl of THF and then 512ng of BMV RNA (Promega, US) in 2µl of water was added and briefly vortexed then incubated for 10min at 22°C. 1µl (10µmol) of acetic anhydride was then added in 20µl of THF containing 180µg of DMAP. The reaction was allowed to proceed for a further 5 min at 22°C and then stopped with 200µl of 70% ethanol and the beads collected by centrifugation at 3000rpm for 5sec and then washed a second time in 70% ethanol and twice in 200µl of water before being resuspended in 20µl of water. The secondary reactant such as acetic anhydride or acetic-formic anhydride could also carry various labels such as 14C or 3H allowing the amount of RNA to be determined using a scintillation measurement of the washed RNA-bead complex. Alternatively, the secondary reactant could be fluorescent such as isatoic or N-methylisatoic anhydrides or carry a label such as biotin permitting quantification of the amount of RNA bound to the bead. The secondary reactant would be expected to modify at least 75% or more of the 2'-OH groups so that the RNA is protected from degradation from for example ribonucleases. However, the secondary modification could also provide a means for a second purification step. For example the secondary reactant could contain a hydrophobic group or a ligand such as biotin for binding to a streptavidin bead. On release from the first solid phase such as BCPB beads the RNA would be specifically bound to a hydrophobic surface such as dodecyl-agarose beads.

Example 5

Probe hybridisation to RNA immobilised on BCPB beads

100ng of BMV RNA (Promega, USA) in 1µl of water was added to 40µl of either THF or DMSO and then 3mg of BCPB beads added, mixed and incubated at 22°C for 15min, the beads were then washed once in 100µl of 70% ethanol, once in 100µl of 10% SDS and three times 100µl of water. The beads were finally resuspended in 50µl of Church hybridisation buffer (0.5M NaPi pH7.2, 7% SDS and 1mM EDTA) containing a 32P dCTP radiolabelled probe complementary to the BMV RNA sequence (RNA 2, nucleotides 1-321). Following incubation for 2 hrs at 55°C, the beads were washed twice in 500µl of 2 X SSC/0.1% SDS to remove non-hybridised probe and then the amount of radioactivity remaining on the beads was determined using a scintillation counter.

The probe can be branched or linear and labelled with a radioactive, fluorescent, coloured, affinity or protein label in a manner identical to standard hybridisation procedures. In this way, the amount of RNA complementary to the probe can be quantitatively determined. This could be useful for example when determining the abundance of transcripts representing specific genes in different tissues. In this case, purified RNA from the tissue of interest could be immobilised onto the BCBP beads and complementary labelled probes hybridised. Unlike a traditional dot blot (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH) the RNA is attached to beads and not a membrane, therefore the kinetics of hybridisation are improved (see Wilkins Stevens et al. (1999) Nucleic Acids Res. 27:1719). Automation of bead handling could be enhanced by the use of paramagnetic particles. Other uses could include diagnostics where a RNA virus from, for example a body fluid such as serum, is immobilised on the BCPB bead or other solid phase

containing 1µl of 6M sodium perchlorate, 1µl of water, 1µl of serum and 1µl (20ng) of radiolabelled RNA was added 1µl of acetyl chloride solution, mixed and incubated at 22°C for 5 minutes before the addition of 3mg BCPB beads. RNA binding efficiency compared with a parallel reaction with no acetyl chloride in two separate experiments indicated that 4.2 and 5.4 times more RNA bound to the beads when the serum had been pre-acetylated. This enhancement of RNA binding may be the result of amino-acids and carbohydrates in the serum becoming acetylated and therefore not competing with the 2'-OH groups of the RNA for the BCPB beads.

Example 15

RT-PCR amplification of RNA purified from serum

100ng of BMV RNA (Promega, USA) in 1µl of water was added to 1µl of fresh human serum and then mixed with 40µl of either THF or DMSO in the presence or absence of 150mM sodium perchlorate or 0.5% SDS (final concentrations) and then 3mg of BCPB beads added, mixed and incubated at 22°C for 16min, the beads were then washed once in 100µl of 70% ethanol, once in 100µl of 10% SDS and three times 100µl of water. The beads were finally resuspended in 8µl of water before addition to the RT reaction.

One half (4µl) of the RNA-BCPB beads were added to a 20µl reverse transcription reaction containing the following final component concentrations: 200 mM Tris-HCl (pH 8.4 at 24°C), 75 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP's, 110ng of BMV-R oligonucleotide primer (GAGCCCCAGCGCACTCGGTC) (SEQ ID NO: 1) and 100 units of

MULV Point Mutant (Promega, USA). The reaction was allowed to proceed for 40 min at 42°C. The cDNA was then used directly in a PCR reaction as follows.

The PCR was carried out in a final volume of 25µl with final concentrations of 15mM Tris-HCl pH 8.8, 60mM KCl, 2.5mM $MgCl_2$, 400 µM each dNTP, 10 pmol of each primer BMV F (CTATCACCAAGATGTCTTCG)(SEQ ID NO: 2) and BMV R (GAGCCCCAGCGCACTCGGTC)(SEQ ID NO: 1) and 1 unit *Taq* DNA polymerase (Amersham Pharmacia Biotech, UK). 2 µl of the bead complex/cDNA reaction was added per reaction. Cycle parameters were 94°C x 10 sec, 55°C x 10 sec and 72°C x 15 sec for 30 cycles. PCR products were visualised following agarose gel electrophoresis and staining with EtBr. It was found that none of the reactions in THF led to an amplification product even if sodium perchlorate or SDS had been added. However, all the DMSO reactions provided a suitable template for RT-PCR, with the addition of 150mM sodium perchlorate or 0.5mM SDS (final concentrations) increasing the amount of PCR product by 4.3 and 11 times respectively. Other successful RT-PCR reactions were obtained from 100ng BMV RNA templates purified from 1µl of serum using 10µl of DMSO and 3mg BCPB beads containing 15 or 150mM sodium perchlorate, or 2% SDS, or 400mM guanidine thiocyanate or 600mM urea. BCPB beads are therefore useful to purify RNA for RT-PCR analysis from serum.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,794,140

DATED : September 21, 2004

INVENTOR : Andrew Simon Goldsborough

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 2,
Lines 59, "et al. (1995)" should read --et al., (1995)--.

Column 3,
Line 32, "(1077)" should read --(1977)--.
Line 50, "conditions it will be should be possible" should read --conditions it will be/should be possible--.
Line 61, "etc" should read --etc.--.

Column 4,
Line 2, "RNAsuch" should read --RNA such--.
Line 32, "pyrrolidonone" should read --pyrrolidone--.
Line 33, "Acids. Res. 25:3925) Capillary" should read --Acids Res. 25:3925). Capillary--.

Column 8,
Line 9, "C_1-C_{36} C_1-C_{36} aminoalkanoyl" should read --C_1-C_{36} aminoaldanoyl--.

Column 9,
Line 52, "heads" should read --beads--.
Line 56, "head" should read --bead--.
Line 59, "of the" should read --to the--.

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UNITED STATES PATENT AND TRADEMARK OFFICE

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PATENT NO. : 6,794,140

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INVENTOR : Andrew Simon Goldsborough

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 10,
Line 20, "reacts the" should read --reacts with the--.
Line 24, "reaction" should read --reactive--.
Line 27, "efficiently" should read --effectively--.
Line 65, "reaction" should read --reactant--.

Column 11,
Line 23, "glass glide" should read --glass slide--.
Line 61, "PCPB" should read --BCPB--.

Column 12,
Line 12, "solid" should read --solvent--.
Line 13, "catalyst" should read --catalysed--.
Lines 62-63, "RNA temperature" should read --RNA template--.

Column 13,
Line 26, "heads" should read --beads--.

Column 14,
Line 4, "30 000 - 100 000 should read --30,000 - 100,000--.
Lines 56-57, "125 M NaCl" should read --125 mM NaCl--.
Line 60, "PCPB" should read --BCPB--.
Line 66, "reactively" should read --reactivity--.

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Column 15,
Line 11, "PCPB" should read --BCPB--.
Line 20, "agel" should read --a gel--.
Line 49, "RNA form" should read --RNA from--.
Line 55, "(C1OC(CH_2)$_2$COC1)" should read --(C1OC(CH_2)$_4$COC1)--.
Line 56, "used is" should read --used to--.

Column 17,
Line 27, "1999 s" should read --1999 as--.
Line 66, "or other" should read --or others--.

Column 19,
Line 47, "betyl-," should read --octyl--.
Lines 63-64, "beads in of 1.7M" should read --beads in 1.7M--.

Column 20,
Line 12, "biding" should read --binding--.
Line 16, "octyl or" should read --octyl to--.

Column 21,
Line 9, "N-methylsatoic" should read --N-methylisatoic--.
Line 25, "Proemga" should read --Promega--.
Line 29, "SDS three times" should read --SDS and three times--.
Line 56, "BDPB" should read --BCPB--.

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Column 26,
Lines 11-12, "(pH 8.4 to 24° C.)" should read --(pH 8.4 at 24° C.)--.
Line 19, "6.0 mM" should read --60 mM--.
Line 30, "perchlorate to SDS" should read --perchlorate or SDS--.

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